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TARGETED EXPRESSION OF A DOMINANT NEGATIVE FGF RECEPTOR IN GnRH NEURONS REDUCES FGF RESPONSIVENESS AND THE SIZE OF GnRH NEURONAL POPULATION

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ABSTRACT

Increasing evidence suggests that fibroblast growth factors (FGFs) are neurotrophic in gonadotropin-releasing hormone (GnRH) neurons. However, the extent to which FGFs are involved in establishing a functional GnRH system in the whole organism has not been investigated. In this study, transgenic mice with the expression of a dominant-negative FGF receptor mutant (FGFRm) targeted to GnRH neurons were generated to examine the consequence of disrupted FGF signaling on the formation of the GnRH system. To first test the effectiveness of this strategy, GT1 cells, a GnRH neuronal cell line, were stably transfected with FGFRm. The transfected cells showed attenuated neurite outgrowth, diminished FGF-2 responsiveness in a cell survival assay, and blunted activation of the signaling pathway in response to FGF-2. Transgenic mice expressing FGFRm in a GnRH neuron-specific manner exhibited a 30% reduction in GnRH neuron number, but the anatomical distribution of GnRH neurons was unaltered. Although these mice were initially fertile, they displayed several reproductive defects, including delayed puberty, reduced litter size, and early reproductive senescence. Overall, our results are the first to show, at the level of the organism, that FGFs are one of the important components involved in the formation and maintenance of the GnRH system.

INTRODUCTION

Neurons that synthesize and release gonadotropin-releasing hormone (GnRH) are central to the initiation and maintenance of reproductive function in vertebrates. Unlike most neurons in the brain, GnRH neurons do not originate within the central nervous system (CNS). In mammals (1, 2), GnRH neurons first become detectable in the olfactory placode region and are thought to originate from this structure; recent work in zebrafish (3) suggests alternative extra-CNS origins for these cells that have yet to be explored in other species. Regardless of their initial origin, after fate specification, GnRH neurons leave their birthplace and subsequently migrate across the cribriform plate to enter the forebrain. Once migration ceases, GnRH neurons target their axons to the external zone of the median eminence (ME) for hormone release (4). Regulation of GnRH neurons at the levels of initial birth and survival, subsequent migration and ultimate axon targeting is crucial, and disruption of any of these developmental phases may result in severe reproductive impairment.

As a first step toward the understanding of how the GnRH system forms, we need to gain insights into key regulatory factors that drive the development and promote the survival of GnRH neurons. Neurotrophic factors have traditionally been implicated in orchestrating a substantial share of these events in the central and peripheral nervous systems (5). As much as neurotrophic factor research has flourished in the recent years, surprisingly little is known concerning the roles these multi-potent signaling molecules play in the formation and maintenance of the GnRH neuronal network. Studying how GnRH neuron development is regulated by neurotrophic factors is critical in gaining further knowledge with regard to the types of signals required to trigger changes in GnRH neuronal physiology.

Fibroblast growth factors (FGFs) are signaling molecules known to have profound neurotrophic effects on the developing nervous system (6). Previously, we reported FGF-2, a

prototypic member of the FGF family, was highly neurotrophic in the immortalized GnRH neuronal cell lines, GT1 cells (7). Recent studies on endogenous GnRH neurons revealed a subpopulation of GnRH neurons expressed FGF receptors (FGFRs; 8). Further, the addition of FGF-2 stimulated neurite outgrowth and the blockade of FGF signaling inhibited fate specification of primary GnRH neurons in culture (8). The clinical relevance of these *in vitro* data was further strengthened by two recent reports indicating a causal relationship between the loss of function mutation in FGF receptor 1 (FGFR1) and Kallmann Syndrome, a pathology characterized by the complete or partial loss of GnRH function and anosmia (9, 10). These results prompted the hypothesis that FGF signaling is critical for the proper formation and maintenance of a functional GnRH system.

To test this hypothesis, we investigated if the disruption of FGFR function in GnRH neurons resulted in the abnormal formation of the GnRH system. We first tested if the overexpression of a dominant negative FGFR mutant (FGFRm; 11, 12) in GT1 cells, a GnRH neuronal cell line (13), was effective in abolishing FGF responsiveness and altering the differentiative properties of these cells. Next, we generated transgenic mice in which the expression of the FGFRm was targeted to GnRH neurons to disrupt FGFR function in a cell-specific manner. We determined if GnRH neuron-specific disruption of FGFR function in these mice led to an aberrantly formed GnRH system and/or altered fertility. Together, these results enable us to determine if FGF signaling is critical, at the organismal level, for the establishment of a neuroendocrine system essential for vertebrate reproduction.

RESULTS

cDNA constructs

The components of the FGFRm cDNA, a truncated FGFR1, are illustrated in Fig. 1A. This cDNA was used to generate CMV-FGFRm (Fig. 1B) for the transfection of the GT1 cells, and G-FGFRm (Fig. 1C) for the generation of transgenic mice. Details of plasmid construction are described in the Materials and Methods.

Transfected GT1-7 cells

GT1-7 cells were stably transfected with CMV-FGFRm (see Fig. 1B) to disrupt the function of endogenous FGFRs. Northern blot analysis using a randomly-primed ^{32}P-labeled FGFR1 probe revealed that all GT1 cells expressed high levels of the endogenous FGFR1 transcript (4.3 kb; Fig. 2). The three GT1 clones (CMV-FGFRm-1, 2, 3) transfected with CMV-FGFRm also expressed high levels of a smaller transcript (1.5 kb) corresponding to the truncated FGFRm. One clone, CMV-FGFRm-2, expressed a smaller splice variant of the endogenous FGFR1 (Fig. 2). Since one of the clones (CMV-FGFRm-3) appeared to express the highest FGFRm to endogenous FGFR1 ratio, this clone was expanded and used for all subsequent biological studies.

CMV-FGFRm-3 cells exhibited very different cellular morphology compared to the CMV-null control cells (Figs. 3A, B). CMV-null cells were elongated and extended neurites toward neighboring cells (Fig. 3A). In contrast, neurite outgrowth was markedly attenuated in CMV-FGFR-3 cells, which appeared flattened and possessed few cellular processes (Fig. 3B). When CMV-null cells were cultured under a serum-deprived condition, FGF-2 at concentrations ranging from 0.1-1 ng/ml significantly enhanced cell survival. However, FGF-2 treatment had no effect on the survival of CMV-FGFRm-3 cells (Fig. 3C).

The disruption of downstream signaling pathway by FGFRm expression was investigated using an in-gel ERK assay (Fig. 4). This assay estimated the ERK activity by measuring the ability of both p42 and p44 ERKs to phosphorylate a substrate, myelin basic protein, embedded within the gel. CMV-null cells responded to the addition of 1 ng/ml FGF-2 with a robust activation of both p42 and p44 ERKs. However, this activation was completely absent in CMV-FGFRm-3 cells (Fig. 4).

Analysis of transgene expression in the *G-FGFRm* mice

Two founder mice (Founders 1 and 5) with the transgene incorporated into the genome were generated by the pronuclear injection of G-FGFRm (see Fig. 1C) into one-cell embryos. Both the *G-FGFRm-1* and *G-FGFRm-5* lines were initially fertile and were bred to homozygosity. To verify the transgene was expressed correctly, northern blot analysis was conducted on total RNA isolated from the forebrain, hindbrain, and the liver (a negative control; 14) of wild-type (WT) and *G-FGFRm* mice. Two endogenous FGFR1 transcripts, approximately 4.3 kb and 1.8 kb, were observed in RNA extracted from the hindbrain and forebrain of WT mice (Fig. 5A). No FGFR1 transcripts were seen in the liver, a negative control (Fig. 5A). In *G-FGFRm-1* mice, in addition to the two endogenous FGFR1 transcripts (4.3 and 1.8 kb), a smaller FGFR1 transcript of approximately 1.5 kb was present only in the forebrain, where GnRH neurons reside (Fig. 5A). This transcript corresponds to the size of the FGFRm observed in the transfected GT1 cells (Fig. 2). The hindbrain RNA was devoid of the FGFRm transcript, consistent with targeting of the transgene to GnRH neurons. Again, the liver did not express any FGFR1 transcript (Fig. 5A).

For *G-FGFRm-5* animals, WT mice also expressed the 4.3 and 1.8 kb transcripts of endogenous FGFR1 (Fig. 5B). However, *G-FGFRm-5* mice expressed two FGFRm transcripts, one approximately 1.5 kb, and one 1 kb. The latter is likely a splice variant of the former. Both FGFRm transcripts were expressed in the forebrain and hindbrain, demonstrating a rather

promiscuous expression pattern that was not tissue-specific. Neither WT nor *G-FGFRm-5* mice expressed FGFRm in the liver (Fig. 5B). Since *G-FGFRm-1* mice expressed the single correct FGFRm transcript, and its expression was restricted to the forebrain, subsequent expression and phenotypic analyses were conducted exclusively with these mice.

The targeting of the transgene to GnRH neurons within the forebrain was verified by *in situ* hybridization. Nine pairs of adjacent sections through the preoptic area (POA) were used for the evaluation of each animal. One section from each pair was hybridized with a probe for GnRH, and one with a probe for the transgene mRNA. The transgene was expressed in approximately 79% of GnRH neurons examined (33 out of a total of 42 GnRH neurons found in 9 sections; Figs. 6A, C) in *G-FGFRm-1* mice. In sections from WT mice, 37 GnRH neurons were identified, and none was positive for the transgene (Figs. 6B, D). There was no visible difference in the distribution of GnRH neurons in brain sections between WT and *G-FGFRm-1* mice. Labeled GnRH neurons were only observed in regions previously described to contain GnRH neurons. There was no ectopic expression of the transgene in other brain regions including the caudate nucleus, cerebellum, cerebral cortex, hippocampus and thalamus in the *G-FGFRm-1* mice (data not shown). Control sense probes did not yield signals above background (data not shown). These findings showed that the transgene expression was specifically targeted to the majority of endogenous GnRH neurons in the *G-FGFRm-1* mice.

Analysis of GnRH neurons in *G-FGFRm-1* mice

To investigate if the targeted expression of FGFRm in GnRH neurons disrupted the formation and/or maintenance of the GnRH system, GnRH immunocytochemistry (ICC) was performed to examine the number and distribution of GnRH neurons in transgenic and WT mice. A GnRH neuronal count revealed a significant reduction ($P < 0.001$) in the GnRH neuron number in *G-FGFRm-1* mice compared to their age-matched controls (Fig. 7). To see if this

phenotype was expressed predominantly in one sex over the other, the group was broken down into males and females. WT males and females possessed 700-800 neurons, a number consistent with the range of GnRH neuron number previously reported for mice (2). A decrease in GnRH neuron number was observed in both male and female *G-FGFRm-1* mice compared to WT controls, although this difference was more pronounced in the males ($P < 0.001$ in males, $P < 0.05$ in females, Fig. 7). Representative photomicrographs showed that the difference between *G-FGFRm-1* and WT male mice was visible at the level of the organum vasculosum of the lamina terminalis (OVLT; Figs. 8A, B). Visually, there was also a small but noticeable decline in GnRH fiber density in the ME of the *G-FGFRm-1* males (Figs. 8C, D). Female *G-FGFRm-1* mice showed a visible but less pronounced difference in the number of GnRH neurons in the region of the OVLT (Figs. 8E, F). Similar to males, *G-FGFRm-1* females exhibited a modest but visually noticeable reduction in the fiber density targeting to the ME compared to WT mice (Figs. 8G, H).

To examine if the loss of GnRH neurons was brain region-specific, the distribution of GnRH neurons in WT and *G-FGFRm-1* mice was analyzed in defined areas. The distribution of GnRH neurons was not significantly different between the *G-FGFRm-1* and WT male mice (Fig. 9). Both showed a typical pattern of GnRH neuron distribution, with fewer GnRH neurons anterior to the OVLT, an abrupt increase near the OVLT, and a gradual decrease posterior to OVLT (Fig. 9). The distribution patterns of GnRH neurons in female WT and *G-FGFRm-1* mice were similar to males (data not shown).

Reproductive phenotype of *G-FGFRm-1* mice

The observation that transgenic mice possessed fewer GnRH neurons suggest their fertility may be disrupted. To verify this, several reproductive parameters were examined in male and female WT and *G-FGFRm-1* mice (Table 1). In adult females, fertility (number of days required to produce one litter) and the duration of estrous cycle were not significantly different between the

WT and transgenic mice (Table 1). However, the size of the first litter was significantly reduced in *G-FGFRm-1* females, although this reduction was not apparent in the subsequent litters (data not shown). The age of the final litter production was also significantly younger in *G-FGFRm-1* adult females (Table 1). In the 30-day-old peripubertal animals, vaginal opening was observed in 100% WT females but none of the *G-FGFRm-1* females, indicating delayed puberty in the latter group (Table 1). A trend towards reduced hypothalamic GnRH concentration was observed in these females, although this was not significant (Table 1). Peripubertal *G-FGFRm-1* males had significantly reduced gonadosomatic index (GSI; [g testes mass/g body mass] x 100) and hypothalamic GnRH concentrations compared to WT males (Table 1). In the peripubertal animals, serum LH levels did not differ significantly between WT and transgenic animals for either males or females (Table 1).

DISCUSSION

Although a growing body of evidence suggests FGF signaling is involved in the neurotrophic regulation of GnRH neurons (7, 8, 15), much of the focus was on the action of FGF at the cellular level using *in vitro* models. The overall consequence of disrupting FGF signaling in GnRH neurons has not been investigated at the level of the organism. In the current study, we employed a strategy in which the expression of FGFRm, verified to disrupt signaling in GnRH cell lines, was genetically targeted to GnRH neurons. Targeted expression of the FGFRm to GnRH neurons resulted in a 30% reduction in the GnRH neuronal population and adversely affected several reproductive parameters. These results provided evidence that FGF signaling is critical for the maintenance of the full GnRH neuronal population into adulthood and consequently, the maintenance of optimal reproductive performance throughout the reproductive life span.

The FGFRm is a truncated murine FGFR1 lacking the intracellular tyrosine kinase domain (16). Upon ligand binding to the receptor, the truncated receptor forms nonfunctional heterodimers with the WT FGFRs 1, 2, and 3, thereby blocking the subsequent signaling pathway. The same FGFRm targeted by the keratin and surfactant C protein promoters was shown to disrupt FGFR functions in mouse skin and lung bud epithelia, respectively (16, 17). The expression of the FGFRm in GT1 cells abolished the ability of transfected cells to extend neurites and respond to FGF-2 with enhanced survival, both well-documented neurotrophic effects of FGFs (6). The diminished neurite outgrowth in FGFRm-stably transfected GT1 cells was likely due to the blunted autocrine response to the endogenous FGF-2 produced at low levels in GT1 cells (7). The loss of neuronal morphology in FGFRm-transfected cells also suggests basal levels of FGF signaling may be required for the maintenance of a neuronal phenotype.

Lastly, the results from in-gel ERK assay support the well-documented mechanism by which FGFRm abolishes signaling pathways downstream of FGFR activation (11).

It is presently unclear if CMV-FGFRm cells secreted altered levels of GnRH in culture. Voigt *et al*. (15) reported that FGF-2 did not significantly affect GnRH secretion in GT1 cells, suggesting FGF-2 was not involved in the acute regulation of hormone release. Interestingly, the same study also reported increased mRNA levels of prohormone convertase 2, a key enzyme required for the initial processing of the GnRH prohormone, in GT1 cells treated with FGF-2 (15). The latter raised the possibility that GnRH neurons, under the long-term disruption of FGF signaling, may process the GnRH prohormone differently, leading to an altered levels of mature peptide release.

Northern blot analysis of tissue RNA from WT and *G-FGFRm* mice demonstrated the presence of two endogenous FGFR1 transcripts in all animals. The 4.3 kb transcript was widely documented and represented the membrane spanning IIIc variant of FGFR1 (18, 19). The 1.8 kb transcript may represent the IIIa variant of the secreted FGFR1 (14). This FGFR1 was found in the brain (14) and may have cross-hybridized with our probe. *G-FGFRm-1* animals also expressed a single FGFRm transcript consistent with the expected size of the transgene in the brain, where GnRH neurons reside. Further examination with *in situ* hybridization verified that the transgene was indeed expressed exclusively in GnRH neurons of *G-FGFRm-1* mice. In contrast to this expected expression pattern, *G-FGFRm-5* animals expressed an additional splice variant of the FGFRm (1.0 kb) and expressed the transgene ectopically in the hindbrain. It is worth mentioning that our initial observations showed that *G-FGFRm-5* animals had a similar reduction in the number of GnRH neurons (data not shown). However, it was impossible to determine if the reduction in GnRH neurons was specific to the expression of the transgene in GnRH neurons or the consequence of the ectopic expression of the transgene.

G-FGFRm-1 mice exhibited an average of 30% reduction in the size of GnRH neuronal population, with the reduction being more prominent in males than females. Interestingly, we found that WT females had significantly fewer GnRH neurons (Fig. 7; $P < 0.05$ by Student's *t*-test) than males. This particular sexual dimorphism has been reported in mice (20) and may have contributed to the sex difference in GnRH neuron reduction observed here. These results were also consistent with our observation that the reduction in hypothalamic GnRH concentrations was more pronounced in transgenic males than females (see Table 1). Although a modest reduction in GnRH fiber density was also observed in the ME of the *G-FGFRm-1* mice, the ability of GnRH axons to target the ME did not appear grossly altered, as a large number of axons still reached the ME. We believe the reduced fiber density was primarily due to the reduction in the number of GnRH neurons. It is also possible that the disruption of FGFR function resulted in the diminished ability of GnRH neurons to branch axons, leading to decreased levels of fiber intensity. Our previous observation that FGF-2 significantly promoted neurite branching in cultures of primary GnRH neurons supports this possibility (8).

The uniform reduction in the number of GnRH neurons in all brain regions of the *G-FGFRm-1* mice suggests the size of the original GnRH neuronal population that migrated into the forebrain may have been reduced. Several explanations could account for this reduction. We previously showed that FGF signaling was required for the specification of GnRH neuronal fate in the olfactory placode (8). In the absence of an FGF signal, the majority of GnRH neurons failed to emerge from their birthplace. However, the expression of FGFRm in *G-FGFRm-1* mice is coupled to the activation of the GnRH promoter, thus one would not expect FGFRm to be expressed and FGFR function disrupted until after GnRH neurons are fully specified. A more plausible explanation for the reduced GnRH neuronal population is that in the absence of FGF signaling, a subpopulation of GnRH neurons failed to survive. To date, few studies have

investigated the survival of GnRH neurons following fate specification. The prevailing view was that 800 GnRH neurons were born in the olfactory placode, and all 800 GnRH neurons survived and developed normally to reach the forebrain. However, an observation made by Wu *et al*. (21) revealed that the GnRH neuronal population actually reached a total of 2000 at embryonic day (E)12.75. This number then declined to 1100 in postnatal animals, and to 800 in adults. This observation suggested that less than 50% of the original GnRH neuronal population survived to adulthood. Moreover, it underscored the importance of survival-enhancing factors in maintaining and determining the final number of GnRH neurons. FGF-2, a potent survival enhancing factor in GT1 cells (7; current study), may be critical in promoting the survival of GnRH neurons, especially during the developmental period when a large number of GnRH neurons vanished in the forebrain.

Despite the absence of approximately 30% of GnRH neurons in the forebrain of *G-FGFRm-1* mice, these animals were initially fertile. This observation is not surprising considering findings in the hypogonadal (*hpg*) mouse. The *hpg* mice harbored a deletion in the GnRH gene that resulted in the inability to synthesize the mature GnRH peptide. The successful transplantation of just 1-3 detectable GnRH neurons was effective in restoring their fertility (22-24). Thus, although reduced, the number of GnRH neurons present in *G-FGFRm-1* mice was clearly sufficient for maintaining reproduction under laboratory conditions. It is important to point out that although these animals reproduced, the level of reproductive activity was reduced compared to controls. In particular, the delayed puberty, a reduction in the size of the first litter, and early reproductive senescence were all clear signs that the function of the GnRH system at the beginning and the end of the reproductive life span has been markedly compromised.

One might question why serum LH levels in transgenic mice remained unchanged despite the reduced GnRH neuron number and content. A recent study (25) reported similar

observations in mice harboring a mutation in carboxypeptidase E and thus could not process GnRH normally. These mice had very low bioactive GnRH concentrations in the hypothalamus, displayed a number of reproductive defects, but had normal circulating LH. One way these animals compensated for low GnRH was by increasing pituitary sensitivity to GnRH (25). Another study on these mice reported similar reduction in thyrotropin-releasing hormone (TRH) due to processing defect, but again, the level of circulating thyrotropin (TSH) was normal (26). These results, together with ours, clearly demonstrated the ability of animals to develop pituitary-level compensatory mechanisms to cope with diminished levels of releasing hormones.

Our current study provides the first evidence, at the level of the organism, that FGF signaling is involved in the maintenance of GnRH neurons. The significant reduction in the number of GnRH neurons may reflect a decrease in the survival of GnRH neurons during development. Several explanations could account for why only 30% of GnRH neurons were affected. First, the extent of FGFR disruption may vary greatly among individual GnRH neurons depending on the ratio of FGFRm to WT FGFR expression. It has been speculated that the FGFRm would have to be expressed at levels five to ten times higher than the WT receptor to completely inhibit signaling (11). Due to the heterogeneous nature of the GnRH neuronal population (27-30), the level of GnRH gene expression is likely to vary among cells. Consequently, the number of FGFRm expressed per cell is likely to vary since the expression of this transgene is driven by the GnRH gene promoter. Furthermore, variations in the number of endogenous WT FGFRs among GnRH neurons could result in different FGFRm to WT FGFRs ratios, leading to unequal degree of disruption. Secondly, FGFR function may be involved in the direct regulation of only a fraction of GnRH neurons. In support of this notion, Gill *et al*. (8) has demonstrated the presence of FGFRs1 and 3 in only 20-60% of embryonic and adult GnRH

neurons. The consequence of disrupting FGFR function would therefore be negligible for GnRH neurons lacking FGFRs or for those expressing functionally insignificant levels of FGFRs.

If not all GnRH neurons require FGF signaling to mature, there must be other regulatory factors capable of supporting the GnRH system. We hypothesize that his redundancy, commonly observed in the developing CNS, is critically important in safeguarding the GnRH system under a disrupted environment. Neurotrophic factors such as insulin-like growth factor (IGF) I (31-36), IGF II (31, 37), and brain-derived neurotrophic factor (38) have all been shown to regulate GnRH neuronal function and may protect the GnRH system from complete obliteration when FGF signaling is disrupted. Interestingly, IGF-I has also been shown to act synergistically with FGF-2 to regulate the function of GT1 cells (35), suggesting the collaborative nature of neurotrophic factor actions. The strong evolutionary need for an excessively robust GnRH system for the successful propagation of species underscores the importance of this redundancy. Species with a GnRH system that could be rendered nonfunctional as the result of a single regulatory anomaly will not survive the test of time. Even under this type of redundancy, our results have unequivocally demonstrated that FGF signaling is one of the important components involved in the formation of a neuroendocrine system critical for vertebrate reproduction.

MATERIALS AND METHODS

Animals

Control and transgenic mice were derived from the mating of C57BL/6J and DBA/2J mice, and their offspring. All mice were housed in the animal facility under a 12L:12D cycle and fed water and rodent chow *ad libitum*. All animal procedures complied with protocols approved by the Institutional Animal Care and Use Committee of the University of California at San Francisco and University of Colorado at Boulder.

Construction of plasmids

The details of the FGFRm cDNA were described previously (16). Briefly, a 1.1 kb FGFRm, a truncated dominant negative murine FGFR1 cDNA (exon IIIc variant) lacking the intracellular tyrosine kinase domain, was ligated downstream of 0.65 kb rabbit β-globin intron (39). The 0.63 kb human growth hormone gene (hGH) polyadenylation (poly A) sequence was inserted at the 3' end of the FGFRm cDNA. The FGFRm expression construct (Fig. 1A) was cloned into the pBluescript KS+ vector (Strategene, La Jolla, CA).

For the stable transfection of GT1 cells, a partial FGFRm construct, containing only the β-globin intron and FGFRm, was excised with Xba I and Bgl II, blunted with Klenow, and ligated into the pRc/CMV expression vector (Invitrogen, Carlsbad, CA). The resulting plasmid (CMV-FGFRm) was linearized for transfections by digestion with Bgl II (Fig. 1B).

For construction of a FGFRm transgene that would target GnRH neurons, a 2.3 kb fragment of the rat GnRH promoter/enhancer region (G) was inserted via an Xba I site upstream of the rabbit β-globin intron of the FGFRm expression construct. The GnRH promoter/enhancer region consists of a fragment from –2987 to –1172 appended to –441 to +104 and contains a 731 bp deletion (13). This GnRH promoter/enhancer fragment was used successfully to target the

expression of SV40 T antigen to mouse GnRH neurons for the generation of GT1 cells (13). For the production of transgenic mice, the resulting plasmid was excised with Kpn I and Sal I to generate a transgenic fragment (G-FGFRm; Fig. 1C) for the pronuclear injection of one-cell embryos.

GT1 cells and transfection

GT1-7 cells (passages 10-23) were maintained in a GT1 medium consisting of the Dulbecco's Modified Eagle's Medium H21 (DMEM:H21; University of California at San Francisco Cell Culture Facility) supplemented with 5% fetal calf serum, 5% horse serum (HyClone, Logan, UT), and penicillin/streptomycin. GT1-7 cells were transfected with linearized CMV-FGFRm or empty vector (CMV-Null) using DOTAP liposomal transfection reagent according to the manufacturer's instructions (Roche, Indianapolis, IN). Stably transfected cells were selected by culturing cells in GT1 medium containing 450 μg/ml G418 (InVitrogen). After one month, single GT1 colonies were isolated from the culture dish with a sterile pipette tip and transferred to a 12-well plate. One clone was selected for cells transfected with the CMV-Null vector and three clones for the CMV-FGFRm.

Cell survival assay

The responsiveness of transfected cells to FGF-2 was examined using a cell survival assay described previously (7). Briefly, transfected cells were serum-starved for 4 days in the absence or presence of various doses of recombinant human FGF-2 (Promega, Madison, WI). Cells were trypsinized on Day 0 (before serum starvation) and Day 4, and counted with a hemocytometer. Percent cell survival was calculated by dividing the cell number at Day 4 by the cell number at Day 0.

In-gel extracellular-regulated kinase (ERK) assay

The ability of FGFRm to disrupt endogenous FGFR function, and thus downstream signaling events, was monitored in the transfected cells by the in-gel ERK assay described previously (40). We examined the activity levels of p42 and p44 ERKs, both of which were robustly activated in GT1 cells by the administration of FGF-2 (7). Details of this assay were described elsewhere (7).

Generation and screening of transgenic mice

The G-FGFRm transgene was injected into fertilized one-cell embryos as described previously (13). The F2 embryos were derived from the mating of C57BL/6J and DBA/2J mice. Animals were screened for the presence of the transgene by polymerase chain reaction (PCR) of the genomic DNA isolated from tail biopsies. For PCR, we used a 5' primer directed to the rabbit β-globin intron and a 3' primer directed to the FGFRm region. Animals positive for the transgene were designated as *G-FGFRm* mice. All *G-FGFRm* mice were bred to homozygosity by crossing heterozygous *G-FGFRm* mice. Homozygosity was confirmed when a test cross between the *G-FGFRm* mouse and a nontransgenic mate generated offspring that were 100% transgenic. The offspring of nontransgenic littermates produced during the heterozygote x heterozygote crosses were used as control WT animals to match the genetic background of *G-FGFRm* mice.

ICC and quantification of GnRH neurons

Three to 6-month-old WT and *G-FGFRm* mice were perfused intracardially with 20 ml of 0.1 M phosphate-buffered saline (PBS) followed by 30 ml of 4% paraformaldehyde in 0.1 M PBS. Brains were dissected and post-fixed in 4% paraformaldehyde overnight at 4^{o}C, and

cryoprotected in 20% sucrose**.** Forty μm floating sections were cut using a cryostat. Sections were washed with 0.5% hydrogen peroxide in 0.1M PBS containing 0.4% Triton X 100 (PBST) for 10 minutes to the quench the endogenous peroxidase activity, rinsed 5 times with PBST, and incubated for 48 hours at 4^{o}C in PBST containing an anti-GnRH antibody (LR-1, a gift of Dr. R. Benoit, Montreal General Hospital; 1:10,000) and 4% normal donkey serum. After incubation, sections were washed with PBST and incubated with a biotinylated donkey-anti-rabbit IgG (Jackson Laboratory, West Grove, PA; 1:200), washed, and incubated with the Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) for 45 minutes. Sections were washed and the immunoreactivity visualized using diaminobenzidine as the chromagen. After the color reaction, sections were washed, mounted on slides, dehydrated through an ascending series (70-100%) of ethanol, cleared in Histoclear (National Diagnostics, Atlanta, GA), and coverslipped.

GnRH neurons were counted under a Nikon Eclipse E800 microscope. Only cells with darkly stained cytoplasm and clear nuclei were scored. To accurately assess the total number of GnRH neurons in a single animal, cells counts were made in every section ranging from the diagonal band of Broca through the ME.

Northern blot analysis of transgene expression in transfected cells and mice

Total RNA isolated from transfected GT1 cells and from tissues of the control WT and *G-FGFRm* mice were subjected to northern blot analysis to detect the expression of the FGFRm transgene. Fifteen μg of total RNA was fractionated on a 1% agarose/formaldehyde gel and transferred onto a nylon transfer membrane (MSI, Westboro, MA) using the capillary blotting method. A randomly-primed ^{32}P-labeled cDNA probe corresponding to the exon IIIc variant of the murine FGFR1 was generated by the NEBlot Kit (New England Biolabs, Beverly, MA). The membrane was hybridized with the FGFR1 probe at 68^{o}C for one hour. The membrane was then washed twice in 2X SSC/0.1% SDS for 15 minutes at room temperature and once in 0.1X

SSC/0.1% SDS for 15 minutes at 65°C. Hybridization signals were visualized by the exposure of the blot to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) or the Packard Cyclone phosphorimager. The northern blot of mouse tissue RNA was stripped in 50% formamide/2X SSPE at 65°C and reprobed for the presence of glyceraldehyde phosphate dehydrogenase (GAPDH), an internal loading control, using the same procedure.

In situ hybridization analysis of transgene expression in GnRH neurons

The specificity of the transgene expression was analyzed by *in situ* hybridization on adjacent 10 μm frozen serial coronal sections from four 2-month-old homozygous *G-FGFRm* and four 2-month-old WT mice. A detailed protocol for the *in situ* analysis was described previously (41). Briefly, a total of 9 pairs of adjacent sections through the POA were used for the evaluation of each animal. Sections were acetylated, dehydrated progressively, and hybridized overnight at 55°C with the probe for either GnRH or the transgene (see below) following the protocol suggested by the manufacturer (Roche). To localize the mRNA for the FGFRm transgene, a 73 bp fragment corresponding to the 5' end of the hGH poly A fragment was used to generate a digoxigenin-labeled antisense riboprobe. This region was transcribed and was contained within the transgene mRNA. GnRH neurons were identified with a 345 bp digoxigenin-labeled antisense riboprobe to the rat GnRH cDNA (42). The sense strand for both probes was used as a control. For each pair of adjacent sections, one section was hybridized with the GnRH riboprobe while the adjacent section was hybridized with the transgene riboprobe. After hybridization, the slides were washed under high stringency and processed directly for detection of the digoxigenin signal. The sections were examined with a Leica DMR photomicroscope under bright-field optics.

Monitoring the reproductive function of female and male WT and transgenic mice

Reproductive function of adult WT and *G-FGFRm* females were assessed by four criteria: the length of the estrous cycle, the mean duration required for the production of one litter, the size of the first litter, and the age of the female when the final litter was produced. The length of the estrous cycle was assessed by vaginal smears obtained daily for two weeks on females between 3-4 months of age. To assess the average duration required to produce a litter, the number of litters produced by females (between 3-8 months of age) in the constant presence of a male was recorded and normalized for the duration. We retired the female breeders when they failed to produce litters for three months. Thus, the final litter was defined as the litter before the breeding female was retired. Pubertal onset was assessed by measuring the incidence of vaginal opening in 30-day-old females, and GSI in 30-day-old males.

Hormone measurements

Hypothalamic GnRH concentrations in WT and *G-FGFRm* males was measured by a GnRH radioimmunoassay (43). Serum luteinizing hormone (LH) was measured by a sensitive sandwich immunoassay described previously (44).

Statistical analysis

Differences among multiple groups were analyzed by the one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test. Difference between two groups was analyzed by the Student's t-test. Difference in percent animals with vaginal opening between WT and transgenic animals was analyzed by the Fisher's exact test. Differences were considered significant when $P < 0.05$.

ACKNOWLEDGMENTS

We thank Amy Choi, Mai Hashimoto, and Jeremy Jones for the expert technical assistance.

TABLE 1. Reproductive parameters of WT and *G-FGFRm-1* female and male mice.

	Females (n = 4-8)		Males (n = 4-6)	
	WT	*G-FGFRm-1*	Control	*G-FGFRm-1*
Fertility (days/litter)	28.3 ± 3.3	25.4 ± 1.3	--	--
Length of estrous cycle (days)	5.6 ± 1.4	4.8 ± 0.6	--	--
Number of pups in the first litter	8.1 ± 0.6	6.4 ± 0.4*	--	--
Age of last litter production (days)	392 ± 14	289 ± 38*	--	--
Percent vaginal opening at 30 days (%)	100 (8/8)	0 (0/8)*	--	--
GSI at 30 days (%)	--	--	0.64 ± 0.02	0.51 ± 0.02*
30-day-old hypothalamic GnRH (pg/μg protein)	1.4 ± 0.3	0.8 ± 0.1	1.3 ± 0.2	0.5 ± 0.1*
30-day-old plasma LH (ng/ml)	0.15 ± 0.01	0.15 ± 0.01	0.19 ± 0.03	0.28 ± 0.07

All values were expressed as mean ± SEM.
* $P < 0.05$ compared to the controls of the same sex.
ND = not detectable. Parameters not measured or not applicable are denoted by --.

LEGENDS

FIG. 1. Schematic diagrams of **(A)** the FGFRm expression construct, **(B)** the CMV-FGFRm plasmid used for the transfection of the GT1-7 cells, and **(C)** the G-FGFRm fragment used to generate the *G-FGFRm* mice. For **(A)**, ATG denotes the translation initiation site; AB denotes the acid box domain; Ig-II and Ig-IIIc denote the second immunoglobulin-like (Ig) domain and the exon IIIc variant of the third Ig domain; TM denotes the transmembrane domain; double vertical lines denote translation stop codons in all three frames. For **(B)**, black boxes indicate the original components of the pRc/CMV expression vector. The expression of FGFRm in this construct is driven by the cytomegalovirus (CMV) promoter. FGFRm is flanked at the 3' region by the bovine GH (bGH) polyadenylation signal and the neomycin-resistant gene (Neo).

FIG. 2. Northern blot analysis of endogenous FGFR1 and FGFRm transcripts in transfected GT1-7 cells. CMV-null, cells transfected with the linearized pRc/CMV vector without the FGFRm insert; CMV-FGFRm1, 2 and 3, three clones transfected with linearized CMV-FGFRm. The larger transcript at 4.3 kb is the endogenous WT FGFR1. The smaller transcript at 1.5 kb is FGFRm. CMV-FGFRm-2 cells also expressed a splice variant of the wild type FGFR1.

FIG. 3. Morphology and FGF-induced survival of CMV-null cells and CMV-FGFRm-3 cells. **(A)** CMV-null cells were highly neuronal in morphology and possessed extensive neurites, whereas **(B)** CMV-FGFRm-3 cells were flattened and possessed very few processes. **(C)** FGF-2 promoted cell survival in CMV-null cells but not in CMV-FGFRm-3 cells. Each bar represents mean $\pm$ SEM from quadruplicate determinations. Dissimilar letters above the bars indicate a significant difference between the groups ($P < 0.05$).

FIG. 4. FGF-2 (1 ng/ml) activated p42 and p44 ERK activity in CMV-null cells but not in CMV-FGFRm-3 cells. Activity of ERKs was visualized by the phosphorylation of myelin basic protein co-polymerized in the gel. Arrows show the positions of p42 and p44 ERKs. Rapid activation of both ERKs was seen 5 minutes after the addition of FGF-2 in control cells.

FIG. 5. Northern blot analysis of endogenous FGFR1 and FGFRm transgene expression in **(A)** WT control and *G-FGFRm-1* mice, and **(B)** WT control and *G-FGFRm-5* mice. F, forebrain; H, hindbrain; L, liver (negative control). In the forebrain and hindbrain, two transcripts (4.3 kb and 1.8 kb) representing the endogenous FGFR1 are indicated by arrowheads. In *G-FGFRm-1* mice **(A)**, a single transcript (1.5 kb) representing the FGFRm was present in the forebrain (arrow) but absent in the hindbrain. In *G-FGFRm-5* mice **(B)**, two transcripts (1.5 kb and 1.0 kb) representing the two variants of the FGFRm were present in both the forebrain and hindbrain (arrows). Liver RNA samples were consistently devoid of endogenous FGFR1 and FGFRm. GAPDH was used as a loading control.

FIG. 6. Localization of transgene expression in GnRH neurons of *G-FGFRm-1* mice by *in situ* hybridization. Messenger RNA for GnRH and the transgene were labeled by specific riboprobes on adjacent serial sections from the POA of *G-FGFRm-1* **(A, C)** and WT mice **(B, D)**. In the *G-FGFRm-1* mouse, eight GnRH neurons **(A)** were labeled, and six of these were also labeled for the transgene **(C)**. Arrows in **(A)** point to GnRH neurons devoid of the transgene mRNA. In the WT mouse, four GnRH neurons **(B)** were labeled while no labeling was observed for the transgene **(D)**. Scale bar = 40 μm.

FIG. 7. Total number of GnRH neurons in the forebrain of adult WT control and *G-FGFRm-1* mice. A significant difference in GnRH neuronal number was observed between WT control and *G-FGFRm-1* mice in males and females combined (**P < 0.001), in males only (**P < 0.001), and in females only (*P < 0.05). Each bar represents mean ± SEM. N = 4 and 5 for WT control and *G-FGFRm-1* females, respectively; n = 5 for both WT control and *G-FGFRm-1* males.

FIG. 8. Representative photomicrographs of GnRH neurons at the plane of the OVLT (A, B, E, F), and GnRH axon terminals in the median eminence (C, D, G, H). **(A-D)** GnRH neurons and terminals in male WT control **(A, C)** and *G-FGFRm-1* **(B, D)** mice. **(E-H)** GnRH neurons and terminals in female WT control **(E, G)** and *G-FGFRm-1* **(F, H)** mice. Overall, GnRH neurons were visibly reduced in both male and female *G-FGFRm-1* mice near the OVLT (compare A to B, E to F), but this reduction was more pronounced in males. A very small reduction in GnRH fiber density in the ME was also observed in both male and female *G-FGFRm-1* mice (compare C to D, G to H). Scale bars = 100 μm.

FIG. 9. The distribution of forebrain GnRH neurons in **(A)** WT control and **(B)** *G-FGFRm-1* male mice. The X axis, from left to right, represents the rostral to caudal forebrain sequence in 40 μm increments. OVLT is used as a landmark and denoted as 0. Negative numbers on the X axis indicate regions rostral to the OVLT, whereas positive numbers indicate regions caudal to the OVLT. Each bar represents mean ± SEM. N = 4 for control mice, and n = 5 for *G-FGFRm-1* mice.

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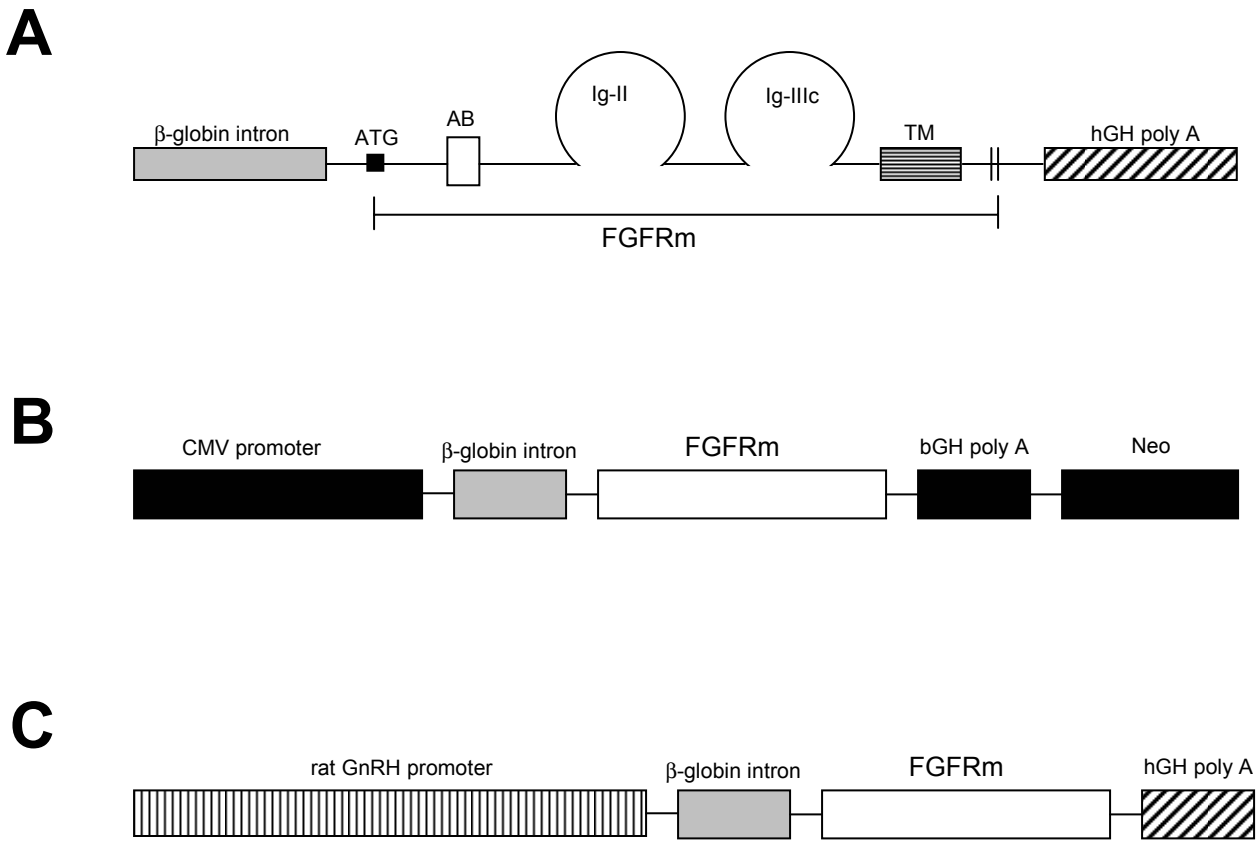
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Fig. 1

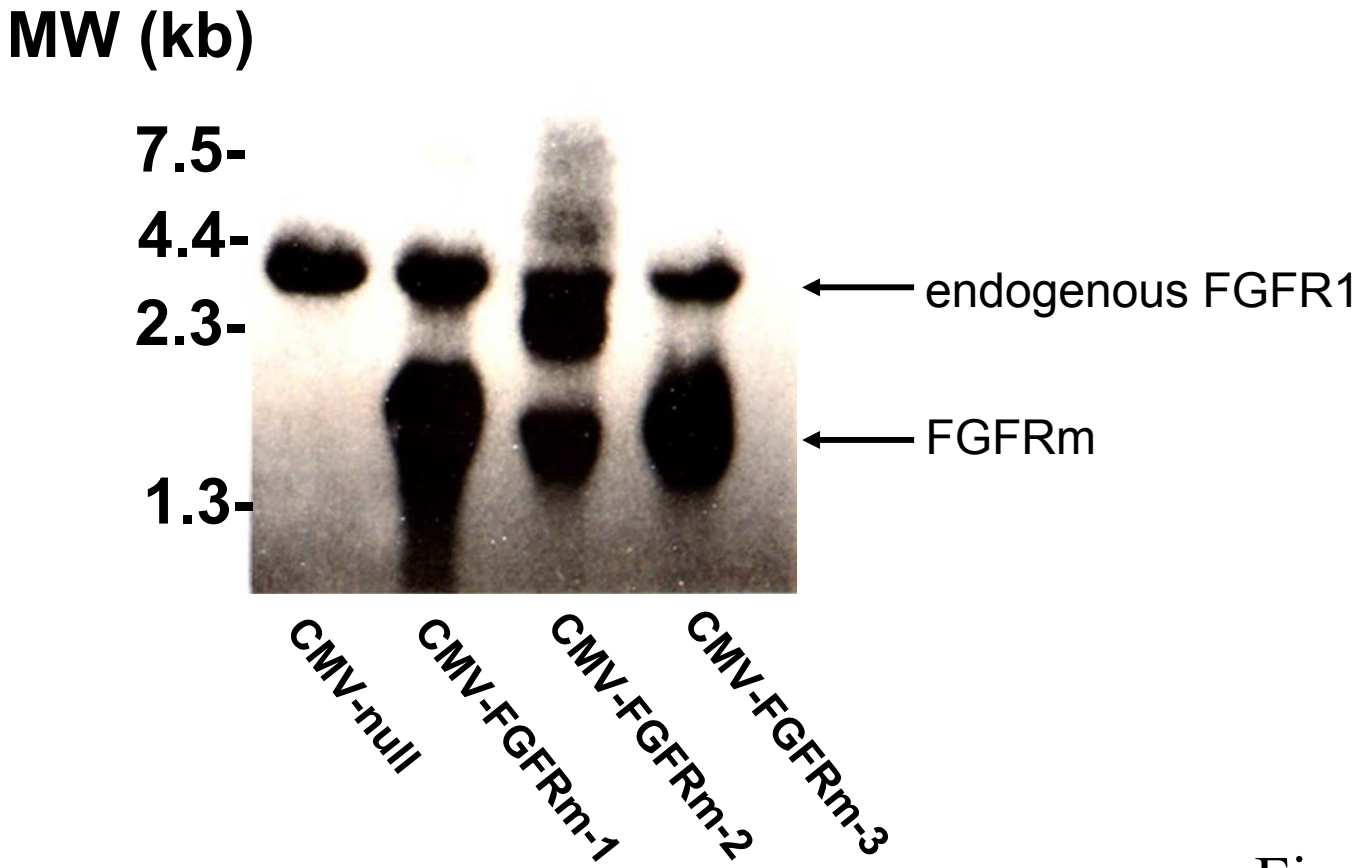
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Fig. 2

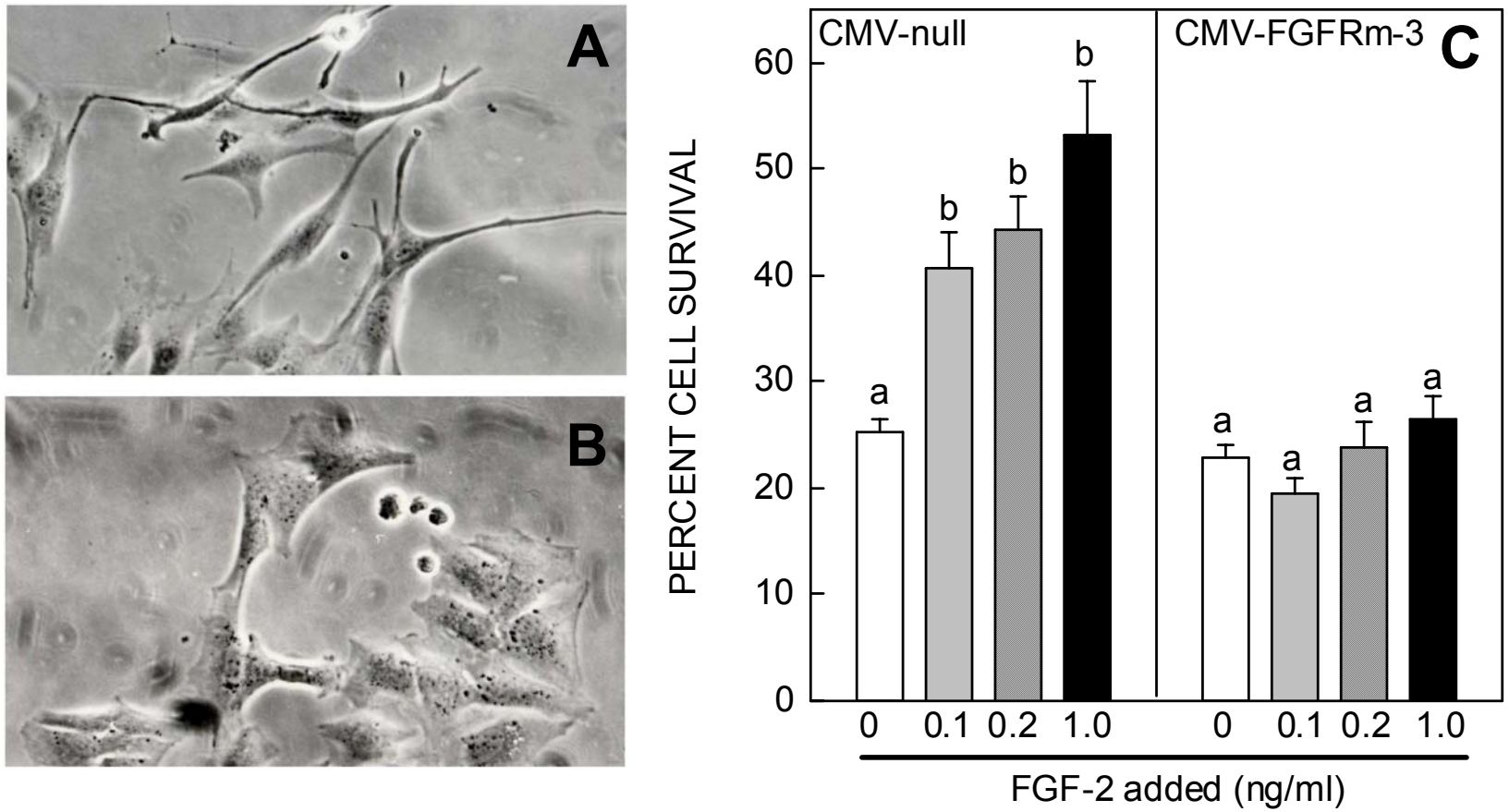
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Fig. 3

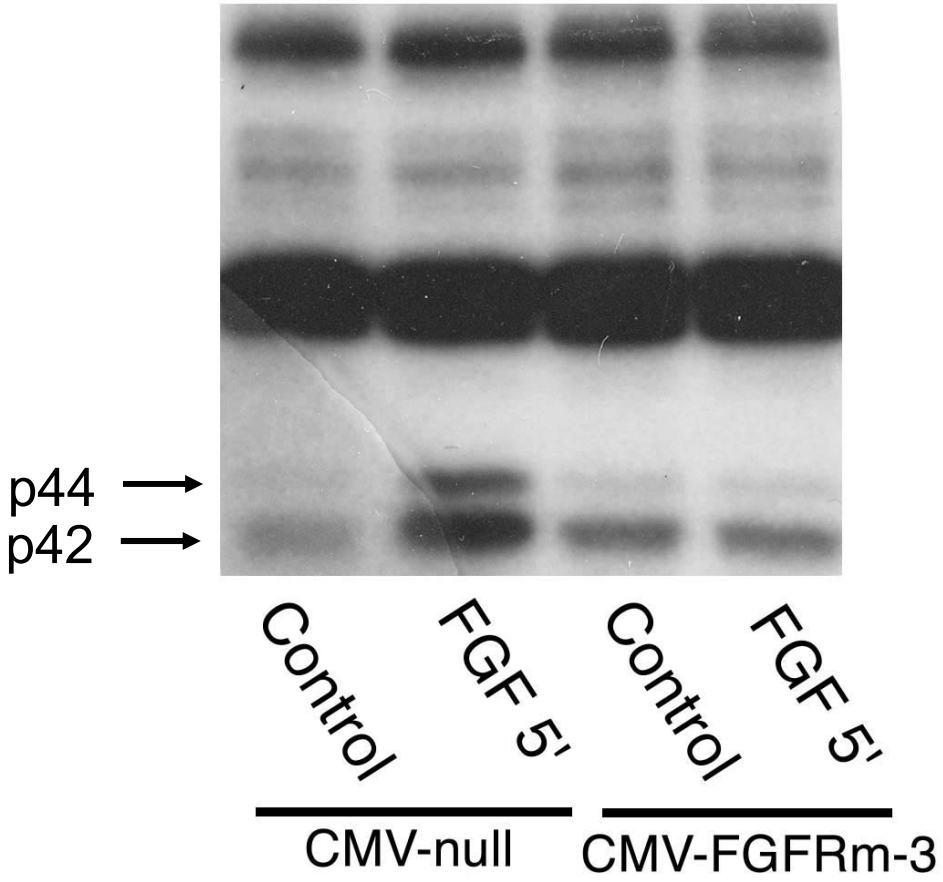

Fig. 4

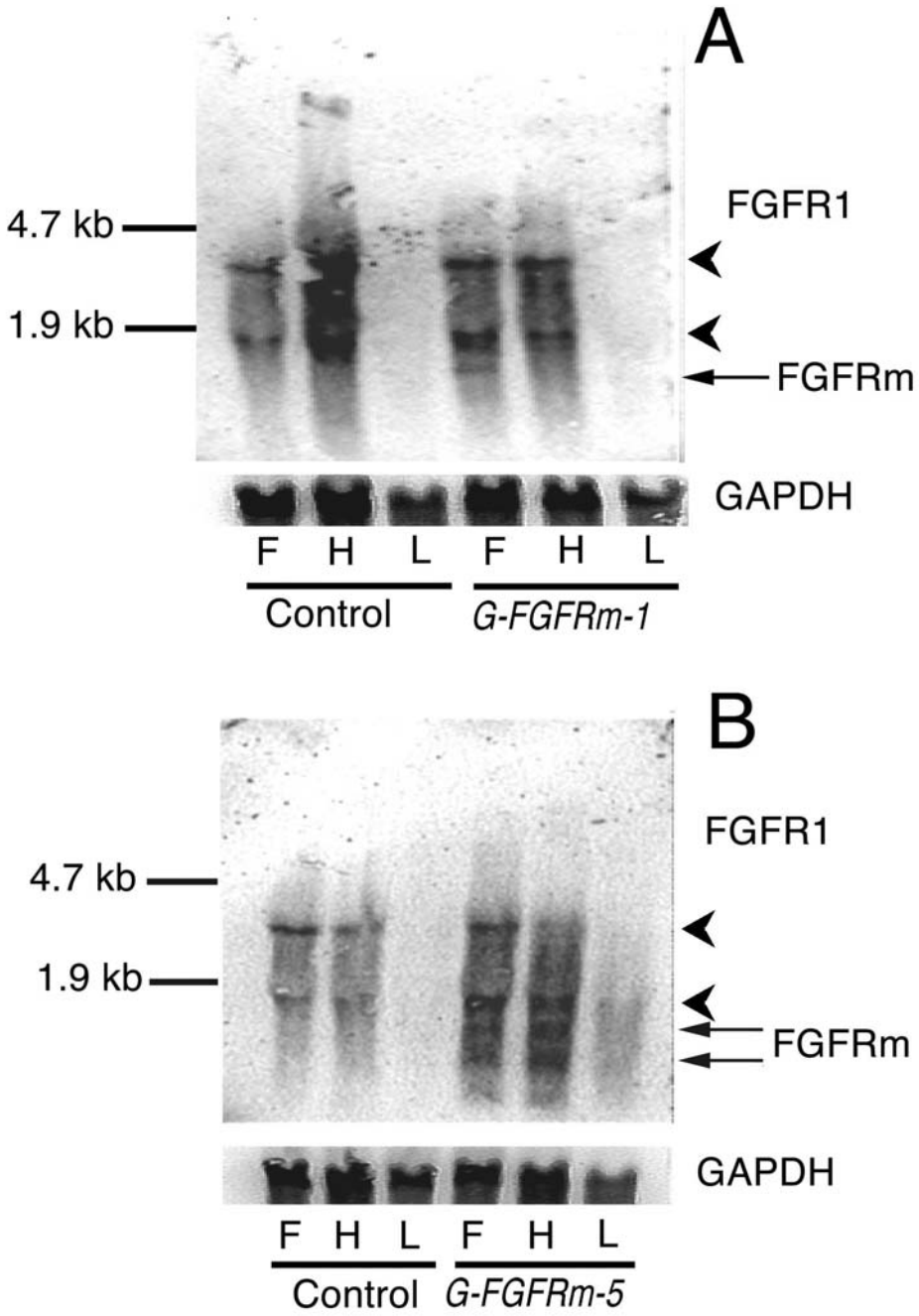
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Fig. 5

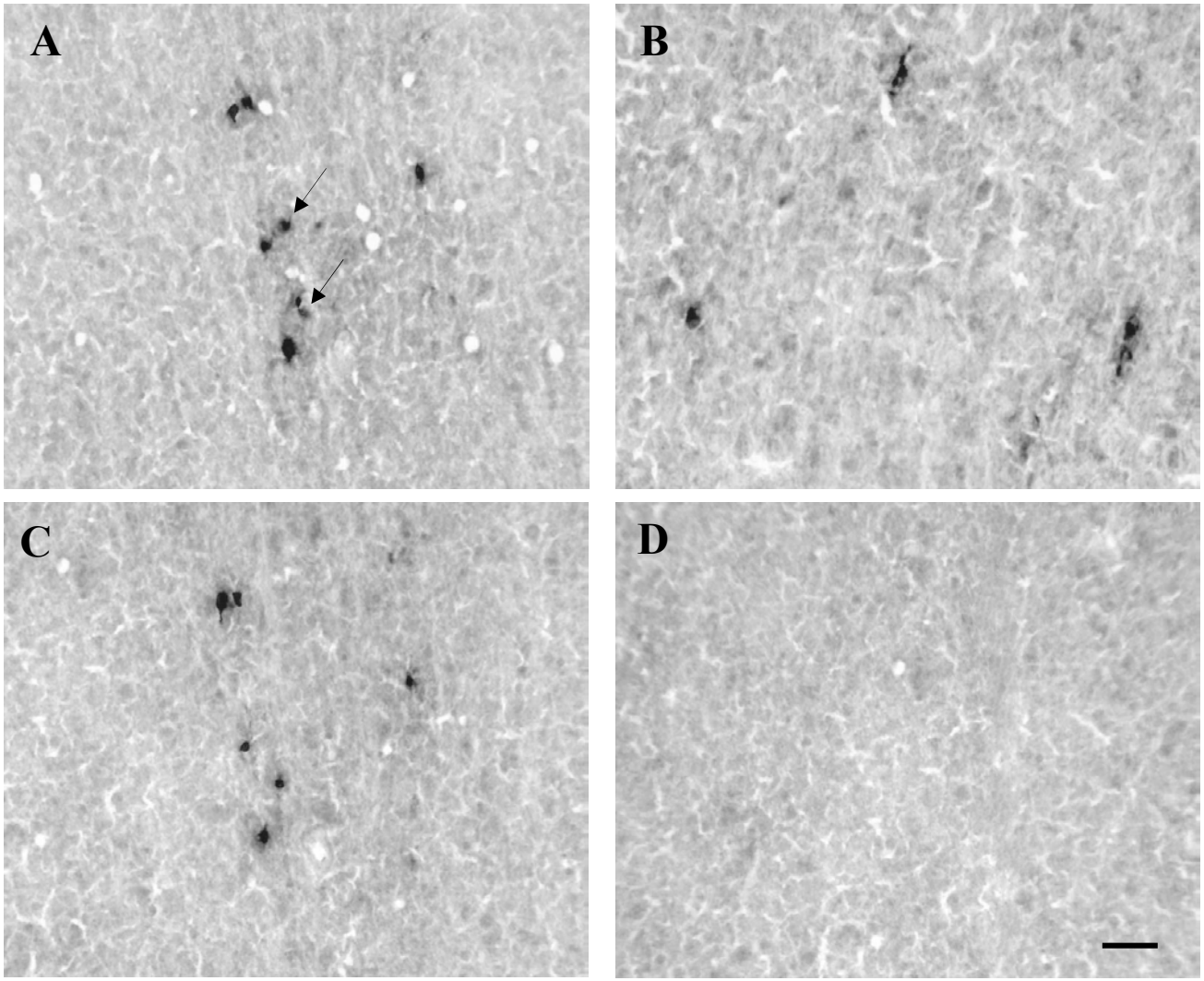

Fig. 6

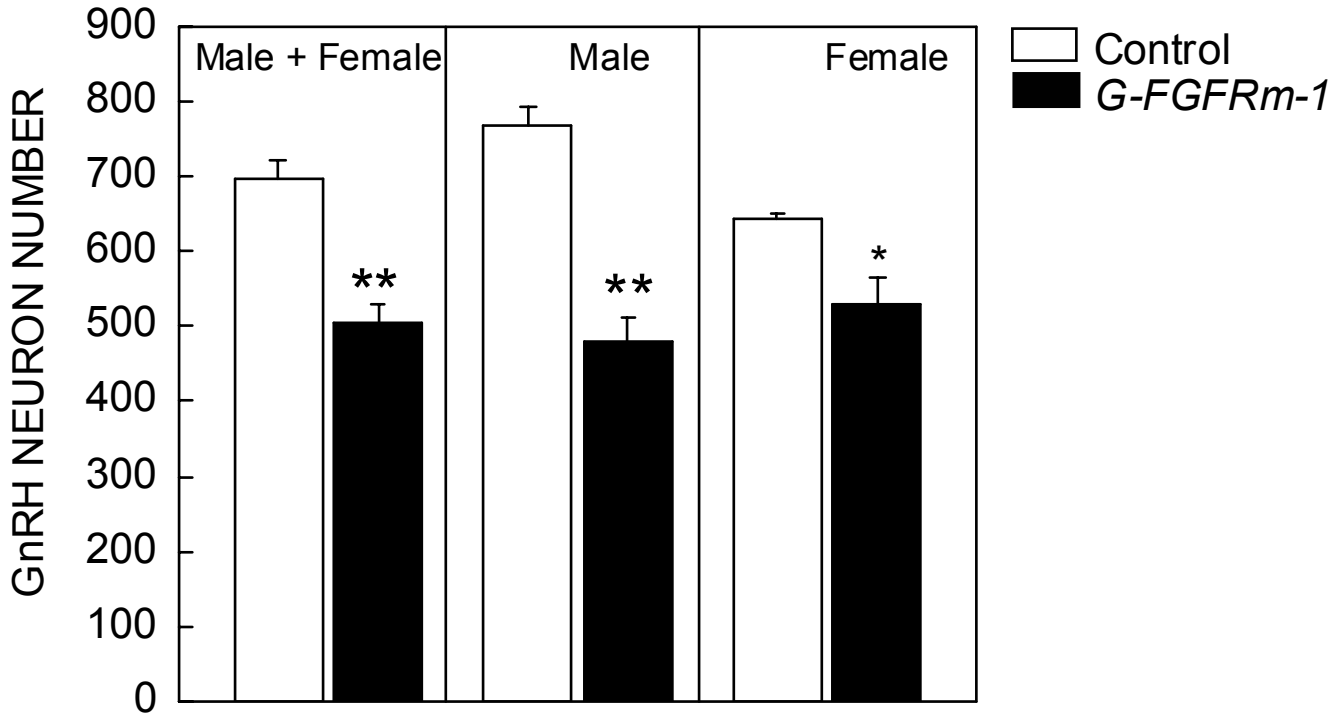
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Fig. 7

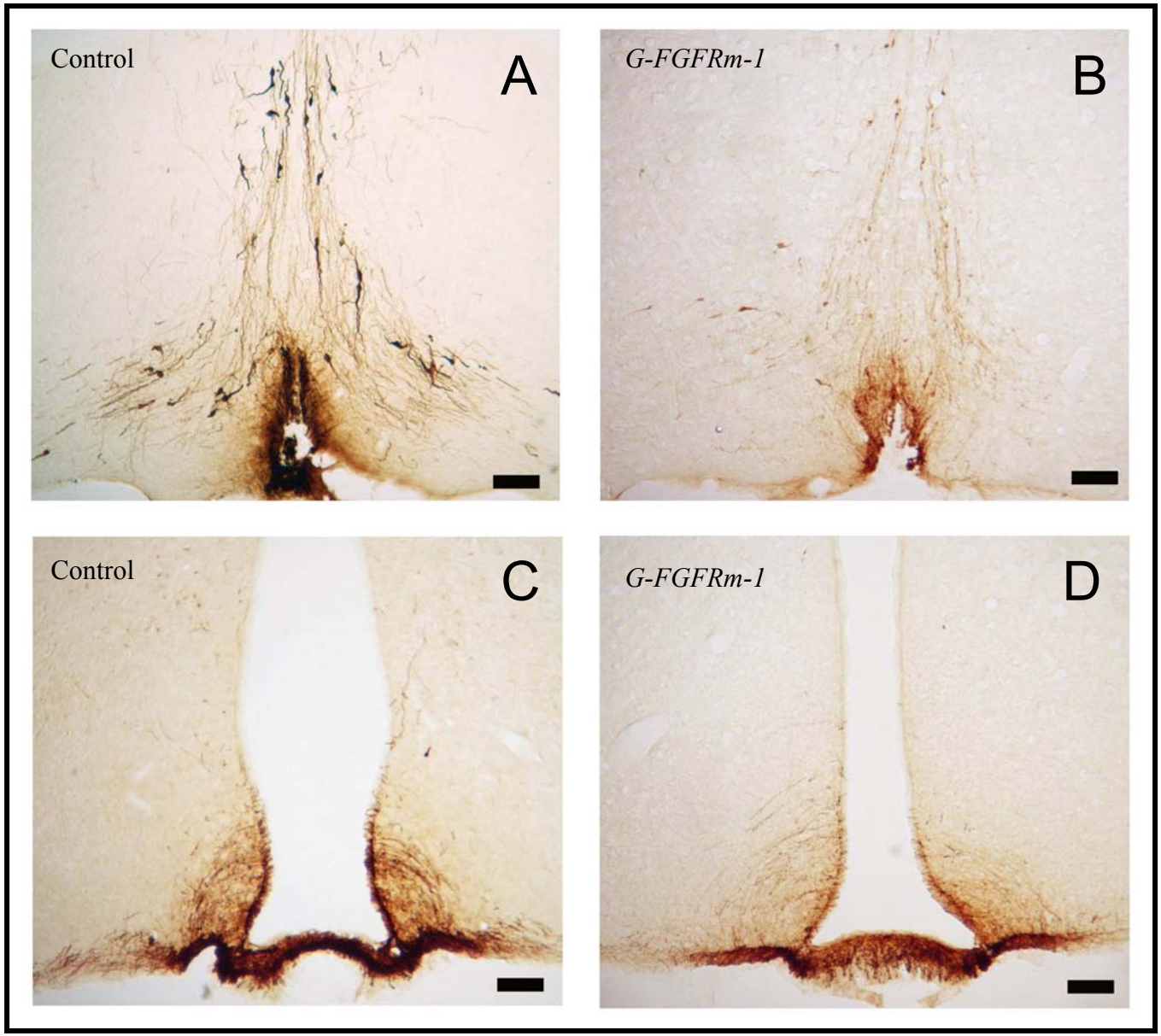

Fig. 8

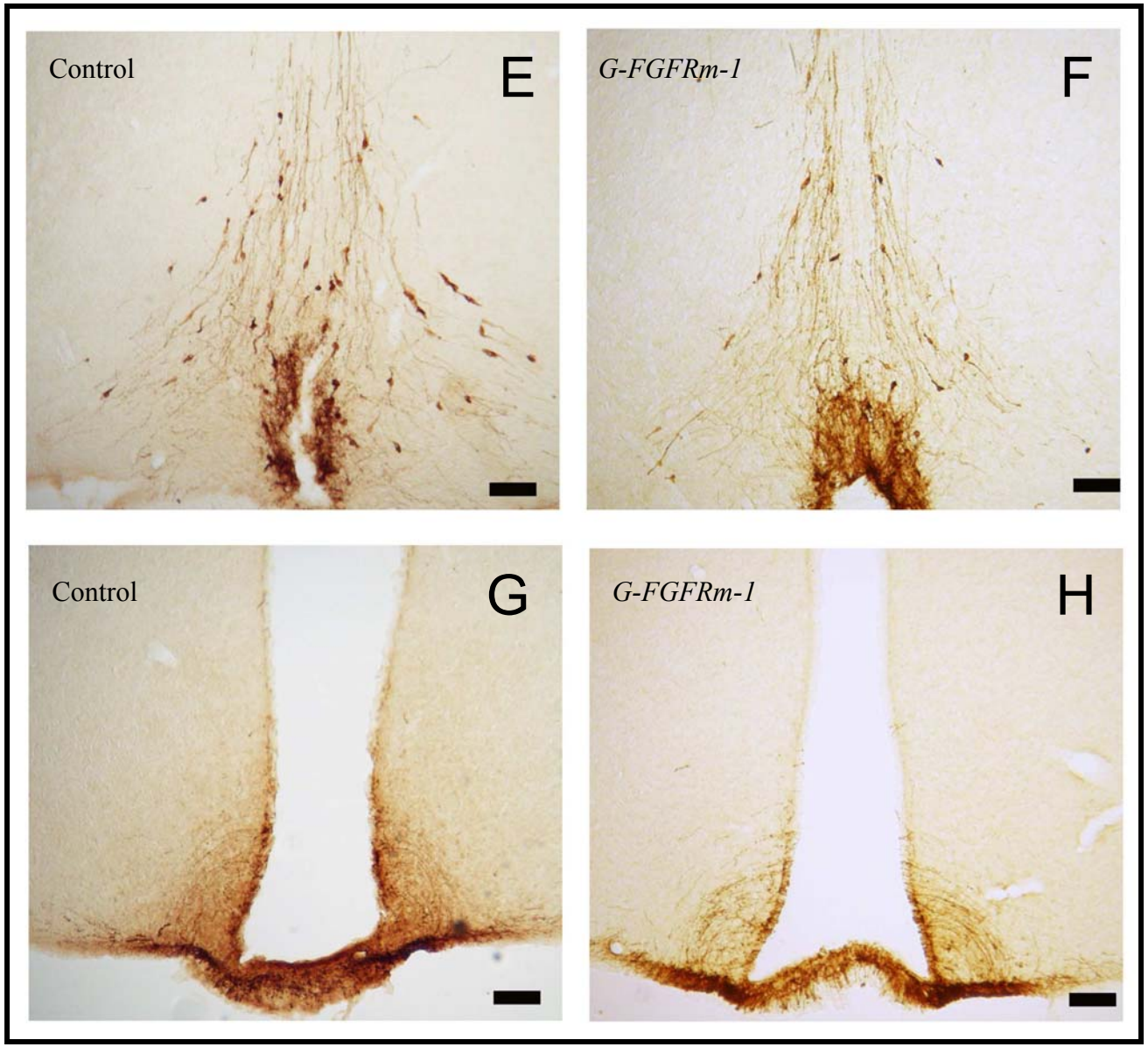

Fig. 8

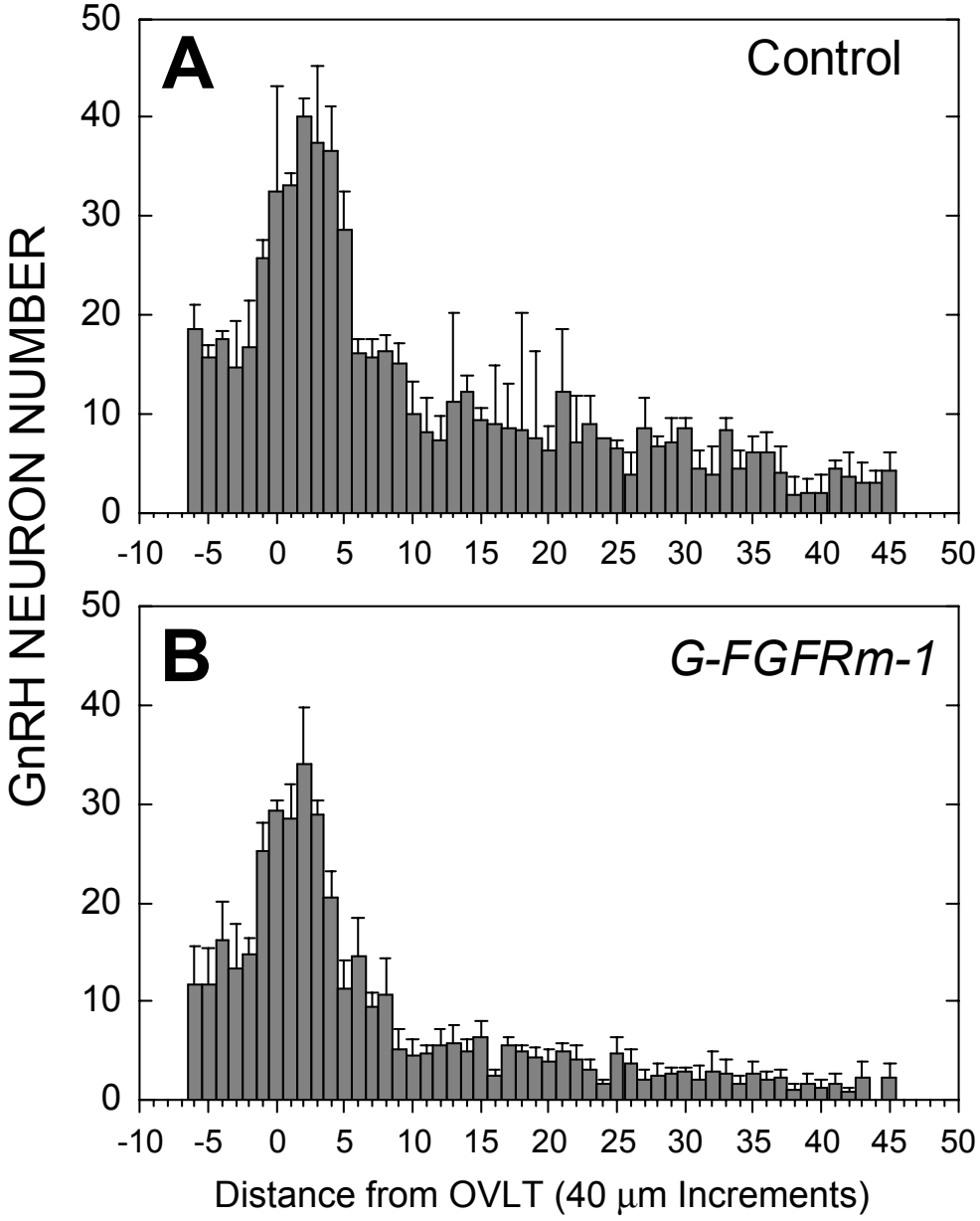

Fig. 9